

**Application
for
United States Letters Patent**

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To all whom it may concern:

Be it known that (we) John F. O'Connor, Galina I. Kovalevskaya, and
Steven Birken

have invented certain new and useful improvements in

METHODS FOR PREDICTING PREGNANCY OUTCOME IN A SUBJECT BY hCG ASSAY

of which the following is a full, clear and exact description.

METHODS FOR PREDICTING PREGNANCY OUTCOME
IN A SUBJECT BY hCG ASSAY

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This application is a continuation-in-part application of U.S. Serial No. 09/017,976, filed February 3, 1998, the contents of which are hereby incorporated by reference into this application.

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The invention disclosed herein was made with United States Government support under National Institutes of Health Grant Nos. NIEHS ES-07589 and HD 15454. Accordingly, the U.S. Government has certain rights in this invention.

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Background of the Invention

Throughout this application, various publications are referenced by author and date. Full citations for these publications may be found listed alphabetically at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art.

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Early pregnancy loss (EPL) is a widespread, but largely undiagnosed problem. In order to adequately diagnose and develop treatments for EPL it is essential to be able to detect and measure the rate of occurrence of EPL. This is critically important in epidemiological studies, some of which are related to exposures to known or suspected reproductive toxins in the workplace, in the environment or by personal use. These early pregnancy losses are often not recognized by women or physicians and are detected solely by the measurement of hCG in the urine at the time between implantation and expected menses. They are sometimes termed "chemical pregnancies" or "occult pregnancies." A landmark epidemiological study established that the incidence of

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EPL was 22% in a population of healthy women attempting to conceive (Wilcox, A. J., et al., 1988). This investigation employed a very sensitive (0.01 ng/ml hCG) assay which detected only the intact hCG molecule with the unique beta subunit carboxyterminal peptide present.

There are multiple likely causes for EPL and clinical spontaneous abortion including genetic abnormality, immunological dysfunction, untreated infection or other unknown physiological problems. In addition, losses may be caused by failure of human chorionic gonadotropin (hCG) to induce adequate response at its target, the corpus luteum. This could result from inadequate hormonal potency. "Nicking" of the beta subunit in the loop 2 region of the molecule, specifically between residues 44-49, can reduce biopotency of hCG. Cleaved peptide bonds in this area of the molecule also exhibit reduced biopotency and reduced immunochemical recognition by monoclonal antibodies directed to the heterodimeric hormone (Cole, L. A., et al., 1991a; Cole, L. A., et al., 1991b; Puisieux, A., et al., 1990; Nishimura, R., et al., 1988; Nishimura, R. T., et al., 1989). Nicked forms of hCG were examined as possibly more prevalent in EPL situations and, at least in part responsible, for early pregnancy loss. Unfortunately many of the reports claiming that substantial concentrations of nicked hCG are produced during pregnancy, losses or successful pregnancies, are not accurate due to faulty assumptions regarding assay specificity. Carbohydrate-modified hCG can also exhibit either reduced or enhanced biopotency. It is known that if the hCG has much reduced sialic acid content and its carbohydrate chains terminate in galactose, much hCG would be removed by the liver receptor for such altered glycoproteins (Braun, J. R., et al., 1996; Kawasaki, T. and G. Ashwell, 1996). The circulating life-time of asialo hCG is reduced and its *in vivo* potency is thereby

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low. Other carbohydrate changes also alter circulating half life; glycoproteins terminating in sulfate-N-acetyl galactosamine are also extracted by a specific liver receptor and have reduced circulating lifetime (Baenziger, J. U., 1994; Fiete, D., et al., 1991).

At least two factors affect increased potency of hCG. First, it is known that a larger Stoke's radius will decrease clearance through the kidney glomerulus which generally clears proteins above an effective size of 70,000 very slowly. The effective size of urinary-isolated hCG is just at this borderline reduced clearance size. Generally, extra sugar content makes the hydrated radius of glycoproteins larger. It has been shown that by adding the hCG beta COOH-terminal peptide to hFSH or hLH, their circulating life-times greatly increased (Fares, F. A. et al., 1992; Matzuk, M. M., 1990). This addition was thought mostly due to the carbohydrate content of that peptide rather than simply the extra polypeptide size. Second, increased negative charge of a protein will prolong its circulating time because of decreased renal clearance (Chmielewski, C. 1992, Quadri, K. H., et al., 1994; Maack, T., et al., 1985). This increased negative charge can be due to extra sialic acid or other negative groups, including sulfate such as is present on hLH and on the pituitary form of hCG (Birken, S., et al., 1996b). Changes which affect signal transduction at the receptor may also affect biopotency of hCG. It is known that deglycosylated hCG has much reduced receptor potency (Ravindranath, N., et al., 1992; Sairam, M. R., and L. G., Jiang, 1992; Browne, E. S., et al., 1990; Sairam, M. R., 1989; Sairam, M. R., et al., 1988). Carbohydrate reduced forms of hCG also have reduced signal transduction (Amano, J., et al., 1990; Bahl, O. P., et al., 1995; Moyle, W. R., 1975).

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The present invention provides a method of predicting pregnancy outcome in a subject by determining the amount of an early pregnancy associated molecular isoform of hCG in a sample comprising: (a) contacting a sample with an antibody which specifically binds to the early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (b) measuring the amount of complexes formed, thereby determining the amount of the early pregnancy associated molecular isoform of hCG in the sample; and (c) comparing the amount early pregnancy associated molecular isoform of hCG in the sample determined in step (b) with the amount determined for temporally matched, normal pregnant subject(s) wherein the relative absence of the early pregnancy associated molecular isoform of hCG in the sample indicates a negative outcome of pregnancy for the subject.

The present invention further provides a method of predicting pregnancy outcome in a subject by determining the amount of an early pregnancy associated molecular isoform of hCG in a sample comprising: (a) contacting a capturing antibody which specifically binds to the early pregnancy associated molecular isoform of hCG with a solid matrix under conditions permitting binding of the antibody with the solid matrix; (b) contacting the bound matrix with the sample under conditions permitting binding of the antigen present in the sample with the capturing antibody; (c) separating the bound matrix and the sample; (d) contacting the separated bound matrix with a detecting antibody which specifically binds to hCG under conditions permitting binding of antibody and antigen in the sample; (e) measuring the amount of bound antibody on the bound matrix, thereby determining the amount of early pregnancy associated molecular isoform

of hCG in the sample; and (f) comparing the amount early pregnancy associated molecular isoform of hCG in the sample determined in step (e) with the amount determined for temporally matched, normal pregnant subject(s), wherein amounts of the early pregnancy associated molecular isoform of hCG in the sample similar to amounts of early pregnancy associated molecular isoform of hCG in temporally matched pregnant samples indicates a positive outcome, amounts of early pregnancy associated molecular isoform of hCG in the sample similar to amounts of early pregnancy associated molecular isoform of hCG in the non-pregnant samples indicates a negative outcome of pregnancy for the subject.

In addition, the present invention provides a method for determining the amount of early pregnancy associated molecular isoforms of in a sample comprising: (a) contacting the sample with an antibody which specifically binds to an early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; and (b) determining the amount of complexes formed thereby determining the amount of early pregnancy associated molecular isoform of hCG in the sample.

Further, the present invention provides a diagnostic kit for determining the amount of early pregnancy associated hCG is a sample comprising: (a) an antibody which specifically binds to an early pregnancy associated molecular isoform; (b) a solid matrix to which the antibody is bound; and (c) reagents permitting the formation of a complex between the antibody and a sample.

The present invention additionally provides an antibody

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which specifically binds to an early pregnancy associated molecular isoform of human chorionic gonadotropin.

5 Further, the present invention provides a method for
detecting non-trophoblast malignancy in a sample
comprising: (a) contacting a sample with an antibody
which specifically binds to the early pregnancy
10 associated molecular isoform of hCG under conditions
permitting formation of a complex between the antibody
and the early pregnancy associated molecular isoform of
hCG; (b) contacting the sample with a second antibody
which specifically binds to intact non-nicked hCG
15 without substantially cross-reacting with said antibody
under conditions permitting formation of a complex
between the antibody and the early pregnancy associated
molecular isoform of hCG; (c) measuring the amount of
complexes formed, thereby determining the amount of the
20 early pregnancy associated molecular isoform of hCG in
the sample; and (d) comparing the amount of early
pregnancy associated molecular isoform of hCG in the
sample determined in step (b) with the amount of early
pregnancy associated molecular isoform of hCG in the
25 sample determined in step (c), wherein a positive
detection of early pregnancy associated molecular
isoform detected in step (b) and a relative absence of
the early pregnancy associated molecular isoform of hCG
detected in step (c) indicates the presence of non-
trophoblast malignancy in the sample.

Finally, the present invention provides a method for detecting gestational trophoblast disease in a sample from a subject comprising (a) contacting a sample with an antibody which specifically binds to the early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular

isoform of hCG; (b) contacting the sample with a second antibody which specifically binds to intact non-nicked hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (c) measuring the amount of complexes formed, thereby determining the amount of the early pregnancy associated molecular isoform of hCG in the sample due to binding with the first antibody, and late pregnancy associated molecular isoform of hCG in the sample due to binding with the second antibody; (d) determining the ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the subject; and (e) comparing the ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the sample determined in step (c) over time, wherein a continuing high ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the sample determined in step (c) indicates the presence of gestational trophoblast disease in the subject.

Brief Description of the Figures

Figure 1.

Bioassay for forms of hCG. This is data from recombinant CHO cells expressing the LH/CG receptor. The response factor is cAMP production. The x-axis is dose of one of four calibrated, pure hormones as described on graph legends. Expressed hCG has no nicks; choriocarcinoma hCG (C5) is 100% nicked; CR 127 was purified into a nick-free (intact) and nick-enriched fraction as shown.

Figure 2.

Incidence (Panel A) and expression level (Panel B) of hCG-related molecules in the positive samples for each of the analyses measured (In early normal pregnancy, n=214; EPL cycles, n=49; and negative cycles, n=297).

Figure 3.

Binding curves for three hCG types in the B152-B207* assay (upper panel) and the B109-B108* assay (lower panel).

Figure 4.

Ratio of hCG isoforms measured by the B152-B207* and B109-B108* assays in normal pregnancy urine (n=103) at different gestational ages. (Regression curve and 95% confidence intervals are shown, $r^2=0.79$). An inflection point in the curve occurs at approximately 29 weeks.

Figure 5.

Box plot of the B152/B109 ratio for pregnancy matched serum/urine at 5-6 weeks of gestational age (n=12); or at 36-39 weeks of gestational age (n=11) and in JAR cell supernatant. Box extends to the 25th and 75th percentile. The upper and lower symbols indicate the 90th and 10th percentile respectively. A solid line inside the box marks the value of the 50th percentile.

Figure 6.

Ratio of hCG isoforms measured by the B152-B207* and B109-B108* assays in the urine of IVF patients (n=65). (Regression curve and 95% confidence intervals are shown, $r^2=0.59$).

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Figure 7.

Immunoassay profiles of fractions from Superose 12 column chromatography of a pooled urine concentrate from pregnant women.

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A method of predicting pregnancy outcome in a subject by determining the amount of an early pregnancy associated molecular isoform of hCG in a sample comprising: (a) contacting a sample with an antibody which specifically binds to the early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (b) measuring the amount of complexes formed, thereby determining the amount of the early pregnancy associated molecular isoform of hCG in the sample; and (c) comparing the amount early pregnancy associated molecular isoform of hCG in the sample determined in step (b) with either (i) the amount determined for temporally matched, normal pregnant subject(s) or (ii) the amount determined for non-pregnant subject(s), wherein the relative absence of the early pregnancy associated molecular isoform of hCG in the sample indicates a negative outcome of pregnancy for the subject. In an embodiment of the present invention, the antibody is B152. Another embodiment of this invention is the early pregnancy associated molecular isoform of hCG.

The hybridoma producing the B152 monoclonal antibody was deposited on February 3, 1998 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. The hybridoma, was accorded ATCC Accession Number HB-12467.

According to one embodiment of this invention, step (a) further comprises a second antibody which specifically binds to hCG without substantially cross-reacting with

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said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG. In an embodiment of this invention, the second detection antibody is

5 B207. According to another embodiment of this invention, step (a) further comprises a second assay antibody B109 which specifically binds to intact non-nicked hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex

10 between the antibody and the early pregnancy associated molecular isoform of hCG. In an embodiment of this invention, the detection antibody is B108. In an embodiment of this invention, step (c) comprises comparing the amount of the early pregnancy associated

15 molecular isoform of hCG determined in step (b) for B152-B207 assay with the amount determined in step (b) for the B109-B108 assay wherein a high ratio of amounts determined for said antibody relative to the second antibody indicates a positive outcome of pregnancy for the subject, a low ratio indicates a negative outcome of

20 pregnancy for the subject.

In yet another embodiment of this invention, step (c) comprises comparing the amount early pregnancy associated molecular isoform of hCG in the sample

25 determined in step (b) with the amount determined for temporally matched, normal pregnant subject(s), wherein amounts of the early pregnancy associated molecular isoform of hCG in the sample similar to amounts of early pregnancy associated molecular isoform of hCG in

30 temporally matched pregnant samples indicates a positive outcome, amounts of early pregnancy associated molecular isoform of hCG in the sample similar to amounts of early pregnancy associated molecular isoform of hCG in the

35 non-pregnant samples indicates a negative outcome of pregnancy for the subject.

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This invention also provides a method of predicting the likelihood of a negative pregnancy outcome in a female subject comprising: (a) contacting a sample from the subject with a capture antibody which specifically binds to an early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (b) contacting any complex formed in step (a) with a labelled detection antibody under conditions permitting binding to the complex the capture antibody and the hCG isoform; (c) measuring the amount of labeled detection antibody bound to the complex so as to thereby determine the amount of the early pregnancy associated molecular isoform of hCG in the sample; and (d) comparing the amount early pregnancy associated molecular isoform of hCG in the sample determined in step (b) with the amount determined for a normal pregnant subject, wherein the relative absence of the early pregnancy associated molecular isoform of hCG in the sample indicates a negative outcome of pregnancy for the subject.

According to an embodiment of this invention, the sample is a urinary sample or a blood sample. In one embodiment of this invention, the sample is an aggregate sample taken from at least one day. In another embodiment, sample may be taken from at least two consecutive days and in a further embodiment, the sample is taken in three days. In an embodiment of this invention, the sample is a spot urine sample, a first morning void urine sample, or an aggregate sample of the first morning void urine samples for at least two consecutive days. In one embodiment of this invention, the antibody is labeled with a detectable marker. In an embodiment of this invention, the detectable marker is a radioactive isotope, enzyme, dye, magnetic bead, or biotin. In a preferred embodiment, the radioactive

isotope is I^{125} .

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The present invention further provides a method of predicting pregnancy outcome in a subject by determining the amount of an early pregnancy associated molecular isoform of hCG in a sample comprising: (a) contacting a capturing antibody which specifically binds to the early pregnancy associated molecular isoform of hCG with a solid matrix under conditions permitting binding of the antibody with the solid matrix; (b) contacting the bound matrix with the sample under conditions permitting binding of the antigen present in the sample with the capturing antibody; (c) separating the bound matrix and the sample; (d) contacting the separated bound matrix with a detecting antibody which specifically binds to hCG under conditions permitting binding of antibody and antigen in the sample; (e) measuring the amount of bound antibody on the bound matrix, thereby determining the amount of early pregnancy associated molecular isoform of hCG in the sample; and (f) comparing the amount early pregnancy associated molecular isoform of hCG in the sample determined in step (e) with the amount determined for temporally matched, normal pregnant subject(s), wherein amounts of the early pregnancy associated molecular isoform of hCG in the sample similar to amounts of early pregnancy associated molecular isoform of hCG in temporally matched pregnant samples indicates a positive outcome, amounts of early pregnancy associated molecular isoform of hCG in the sample similar to amounts of early pregnancy associated molecular isoform of hCG in the non-pregnant samples indicates a negative outcome of pregnancy for the subject.

An embodiment of this invention further comprises (a) removing of the sample from the matrix; and (b) washing the bound matrix with an appropriate buffer. In one

embodiment of this invention, the capturing antibody is B152. In one embodiment of this invention, the detecting antibody is B207. In an embodiment of this invention, step (a) further comprises a second capturing
5 antibody which specifically binds to intact non-nicked hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG. According to an
10 embodiment of this invention, the second capturing antibody is B109 and the second detection antibody is B108. In an embodiment of this invention, step (d) further comprises a second detecting antibody which specifically binds to hCG without substantially cross-
15 reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG. In an embodiment of this invention, step (f) comprises comparing the amount of the early pregnancy associated
20 molecular isoform of hCG determined in step (e) for said antibody with the amount determined in step (b) for the second antibody, wherein a high ratio of amounts determined for said antibody relative to the second antibody indicates a positive outcome of pregnancy for
25 the subject, a low ratio indicates a negative outcome of pregnancy for the subject.

According to an embodiment of this invention, the sample is a urinary sample or a blood sample. In one
30 embodiment of this invention, the sample is an aggregate sample taken from at least two consecutive days. In an embodiment of this invention, the sample is a spot urine sample, a first morning void urine sample, or an aggregate sample of the first morning void urine samples
35 for at least two consecutive days. In one embodiment of this invention, the antibody is labeled with a detectable marker. In an embodiment of this invention,

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the detectable marker is a radioactive isotope, enzyme, dye, magnetic bead, or biotin. In a preferred embodiment, the radioactive isotope is I^{125} .

5 In addition, the present invention provides a method for determining the amount of early pregnancy associated molecular isoforms of in a sample comprising: (a) contacting the sample with an antibody which specifically binds to an early pregnancy associated
10 molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; and (b) determining the amount of complexes formed thereby determining the amount of early pregnancy associated
15 molecular isoform of hCG in the sample.

According to an embodiment of this invention, the antibody specifically binds a region of the early pregnancy associated molecular isoform of hCG comprising
20 a carbohydrate moiety. In one embodiment of this invention the antibody is produced by a hybridoma cell line. In one embodiment of this invention the antibody is B152.

25 Further, the present invention provides a diagnostic kit for determining the amount of early pregnancy associated hCG is a sample comprising: (a) an antibody which specifically binds to an early pregnancy associated molecular isoform; (b) a solid matrix to which the
30 antibody is bound; and (c) reagents permitting the formation of a complex between the antibody and a sample. In an embodiment of this invention, the antibody is B109 or B152. An embodiment of this invention further comprises control sample(s) normal
35 pregnant sample(s), nonpregnant sample(s), or male sample(s). The kit may also contain detection antibodies such as B207 or B108.

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According to an embodiment of this invention, the sample is a urinary sample or a blood sample. In one embodiment of this invention, the sample is an aggregate sample taken from at least one or may be two or three consecutive days. In an embodiment of this invention, the sample is a spot urine sample, a first morning void urine sample, or an aggregate sample of the first morning void urine samples for at least one, or may be two or three consecutive days. In one embodiment of this invention, the antibody is labeled with a detectable marker. In an embodiment of this invention, the detectable marker is a radioactive isotope, enzyme, dye, magnetic bead, or biotin. In a preferred embodiment, the radioactive isotope is I¹²⁵.

The present invention additionally provides an antibody which specifically binds to an early pregnancy associated molecular isoform of human chorionic gonadotropin.

In an embodiment of this invention, the antibody specifically binds to a region of the early pregnancy associated molecular isoform of human chorionic gonadotropin comprising a carbohydrate moiety.

According to one embodiment of this invention, the monoclonal antibody is B152. In an embodiment of this invention, a hybridoma cell (ATCC Accession No. HB-12467) is provided capable of producing monoclonal antibody B152. Another embodiment of this invention is the early pregnancy associated molecular isoform of hCG recognized by the B152 monoclonal antibody.

Further, the present invention provides a method for detecting non-trophoblast malignancy in a sample comprising: (a) contacting a sample with an antibody which specifically binds to the early pregnancy associated molecular isoform of hCG under conditions

detectable marker. In an embodiment of this invention, the detectable marker is a radioactive isotope, enzyme, dye, magnetic bead, or biotin. In a preferred embodiment, the radioactive isotope is I^{125} .

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Finally, the present invention provides a method for detecting gestational trophoblast disease in a sample from a subject comprising (a) contacting a sample with an antibody which specifically binds to the early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (b) contacting the sample with a second antibody which specifically binds to intact non-nicked hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (c) measuring the amount of complexes formed, thereby determining the amount of the early pregnancy associated molecular isoform of hCG in the sample due to binding with the first antibody, and late pregnancy associated molecular isoform of hCG in the sample due to binding with the second antibody; (d) determining the ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the subject; and (e) comparing the ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the sample determined in step (c) over time, wherein a continuing high ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the sample determined in step (c) indicates the presence of gestational trophoblast disease in the subject.

In an embodiment of this invention, the antibody is B152

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or B109. In another embodiment of this invention, the detection antibody is B108 for B109 assay, B207 for B152 assay. In an embodiment of the present invention, the gestational trophoblast disease is choriocarcinoma or hydatidiform mole.

According to an embodiment of this invention, the sample is a urinary sample or a blood sample. In one embodiment of this invention, the sample is an aggregate sample taken from at least two consecutive days. In an embodiment of this invention, the sample is a spot urine sample, a first morning void urine sample, or an aggregate sample of the first morning void urine samples for at least two consecutive days. In one embodiment of this invention, the detection antibody B207 or B108 is labeled with a detectable marker. In an embodiment of this invention, the detectable marker is a radioactive isotope, enzyme, dye, magnetic bead, or biotin. In a preferred embodiment, the radioactive isotope is I^{125} .

As described herein below, unexpected isoforms of hCG are produced during normal early pregnancy. Using an in vitro bioassay, it appears that these isoforms have enhanced potency for signal transduction. These isoforms can be measured using the novel sensitive, immunoassay described herein. This can help predict pregnancy outcome where one cause of early pregnancy loss is failure to produce the isoform of hCG of higher potency produced by successful pregnancies. This enables physicians to intervene to sustain a failing pregnancy. Identification of the nature of the hCG isoform required might provide the proper reagent needed to sustain pregnancy.

New antibodies for measurement of nicked forms of hCG described herein below were developed based on the hypothesis that forms of hCG, which have greatly reduced

bioactivity, contribute to early pregnancy loss (EPL), due at least in part to diminished biopotency. Evidence was found that the hCG that appears in EPL patients displays reduced biological activity. However, it was determined that the cause of the reduced bioactivity is not the presence of nicked hCG in EPL patients. Instead, the hypothesis is that patients that carry pregnancies forward produce an isoform of hCG with enhanced bioactivity. The instant invention describes a unique immunochemical assay to measure this unexpected and previously un-characterized isoform of early pregnancy hCG directly in clinical samples of blood and urine. One of the antibodies developed reacted against a nicked form of hCG isolated from a choriocarcinoma patient, was not specific for a nicked form of hCG but appeared to discriminate among carbohydrate variants of hCG. This antibody, designated B152, appears to preferentially bind hCG forms from choriocarcinoma patients. In studying the content of hCG isoforms during pregnancy, the unique and unexpected observation was made that B152 in the first four weeks of pregnancy measured much higher quantities of an isoform of hCG as compared to the standard hCG isoforms measured by the usual heterodimeric hCG assays exemplified by a previously described B109 based assay. In fact, in early pregnancy (days 9,10,11 postovulation) B152 measured as much as 20-fold more hCG, than did another monoclonal antibody, B109. Later in pregnancy, the B152 isoform declines and is lower in third trimester pregnancy urine than the standard isoforms measured by B109. A further striking observation was that in very early pregnancy, a high B152/B109 ratio correlates with a successful pregnancy outcome while a low ratio correlated with pregnancy loss. This discovery is important as the potentially overlooked isoforms of hCG described herein during pregnancy may be predictors of successful pregnancy outcome. Such an assay has wide

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medical applications and provides a clinician with opportunity to intervene very early in pregnancy if the assay indicated that the pregnancy appeared troubled.

- 5 An antibody, designated B152, produced by the hybridoma cell accorded ATCC Accession number HB-12467 generated against a nicked form of hCG isolated from a choriocarcinoma patient, but not specific for nicked isoform hCG is able to discriminate among carbohydrate variants of hCG. B152 is specific for an early pregnancy associated molecular isoform of hCG. which in the first four weeks of pregnancy is measured at much higher quantities than the hCG standard isoforms measured by the usual heterodimeric hCG assays exemplified by a previously described B109 based assay. Later in pregnancy, the B152 isoform declines and is lower in third trimester pregnancy urine than the standard isoforms measured by B109.
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- 20 This invention is illustrated in the Experimental Details section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.
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EXPERIMENTAL DETAILS

Example 1: Analysis of Molecular Isoforms of hCG in
Early Pregnancy and Early Pregnancy Loss

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Introduction

Almost all investigations of the incidence of early pregnancy loss (EPL), either in normal populations or in populations at risk as a consequence of exposure to putative reproductive toxins (Hakim, R. B., et al., 1995; Lasley, B. L., et al., 1995) use assays for heterodimeric, non-nicked hCG or combination assays which additionally include free beta subunit and beta core fragment of hCG. One concern about the forms of hCG to include in the measurement in EPL was heightened with respect to the nicking phenomenon described above. Because nicked hCG molecules are not measured by the antibodies employed in most EPL studies, the incidence of EPL is presumably underestimated by an amount proportional to the extent of nicking in the urinary molecule. Another concern of significant importance was a determination of the nature of the "hCG like" immunoreactivity in the urine in the periovulatory surge of the menstrual cycle (O'Connor J., et al., 1995). Recent reports have confirmed the existence of and documented the structure of a sulfated form of hCG produced in the pituitary (Birken, S., et al., 1996b). There is a pulsatile secretion of hCG in both men and non-pregnant women. (Odell, W. D.; Griffin, J., 1989 and Odell, W. D.; Griffin, J., 1987). The presence of a non-pregnancy associated form of sulfated hCG of pituitary origin, peaking at ovulation and perhaps persisting into the luteal phase, could potentially interfere with the accurate estimation of EPL.

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Unappreciated isoforms of hCG in blood and urine very early in pregnancy may be more potent *in vivo* than the forms of hCG produced later in pregnancy. The absence

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of such isoforms may be one cause of early pregnancy loss. A sensitive and specific immunoassay system was designed and made to measure unique early pregnancy associated molecular isoforms (EPMI) of hCG. These isoforms, likely to differ by carbohydrate composition, are predictive of a successful pregnancy outcome. When these early pregnancy associated molecular isoforms of hCG are absent or present in low concentration, the pregnancy may be lost very early and be observed as only a "chemical" pregnancy. These hCG isoforms may resemble the forms of hCG produced in some choriocarcinoma patients from which the immunogen used to produce monoclonal antibody B152 was derived as described herein below. The isoforms resemble those from trophoblastic disease not in terms of nicking or intact peptide chains but likely in carbohydrate content. The present invention describes that the molar ratio of B152 to B109 epitopes are predictive of a successful pregnancy or a loss. Three categories of pregnant patients were analyzed: (a) normal pregnant women, (b) women who experience recurrent abortions, (c) women undergoing embryo implantation.

patients). In addition, specimens from gestational trophoblastic disease (GTD) can be used to discriminate between GTD and normal pregnancy.

Results

5 In vitro bioassay for hLH/hCG

An hCG bioassay was constructed employing CHO cells expressing functional human LH/CG receptor. Figure 1 illustrates the differences in vitro in biological activity between nicked and non-nicked hCG as measured by this assay. This system, has been used to evaluate the activity of pituitary and placental hCG (Birken, S., et al., 1996b). Preparations of hCG were tested for nicked and non-nicked molecular isoforms of hCG in a second recombinant bioassay system (Ho, H-H., et al., 1997). Similar results were obtained in both systems.

Normal pregnancy values compared with EPL values.

Figure 2 indicated that nicked hCG is not a significant molar constituent of either early pregnancy or EPL. Data indicated that biological activity is not correlated with nicked hCG, but is instead ascribed to a form of hCG recognized by the B152 monoclonal antibody -- an early pregnancy associated molecular isoform of hCG (EPMI hCG). It has been established that there is diminished hCG bioactivity associated with EPL as compared to early normal pregnancy (Ho, H-H., et al., 1997). Thus, diminished hCG biological activity is a factor in EPL as a consequence of a heretofore unappreciated isoform of hCG - an early pregnancy associated molecular isoform of hCG.

hCG Urinary Analytes. Metabolites of hCG and hLH were studied in a variety of states (Birken, S., et al., 1996a). One study indicated a 31% pregnancy loss (Zinaman, MJ, et al., 1996) while another indicated a 17.4% rate of early pregnancy loss based on hCG assays (Ellish, N. J., et al., 1996). It is known that hCG and

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hCG beta core can be readily transferred from the uterus to the circulation even in the absence of implantation (Chang, P. L., 1997). The molecular spectrum of hCG urinary analytes in EPL cycles, normal conceptive cycles and non-conceptive cycles has been evaluated. The study design and demographics of the investigation have been described (Ellish, N. J., et al., 1996).

Briefly, three urine specimens per cycle, corresponding to days 9,10, 11, post calculated day of ovulation were collected and analyzed in a screening assay (the "combo") which simultaneously detects intact, non-nicked hCG, hCG free beta subunit, and hCG beta core fragment. Individual determinations for each of these analytes, as well as for nicked hCG, and the form of intact hCG detected by monoclonal antibody B152 (EPMI hCG) were performed on these specimens. In addition, since the concentration of luteal phase hLH urinary analytes is a concern because of cross-reaction in hCG assays, levels of intact hLH, hLH free beta subunit and hLH beta core fragment were determined in the normal pregnancy cycles and the non-conceptive cycles. Table I summarizes the characteristics of immunometric assays employed.

TABLE 1. Assay format and specificity

<i>Assay format</i>	<i>Primary analyte</i>	<i>% cross-reactivity with related analytes</i>	<i>Intra-assay cv, %</i>	<i>Inter-assay cv, %</i>
B109-B108*	intact non-nicked hCG	<1% ^b	6	12
B201-C104*	hCG free beta subunit (non-nicked+nicked)	1% hCG; 10% hCG nicked (pregnancy); <1% ^b	6	12
B210-B108*	hCG beta core fragment	2% hLH beta core fragment; <1% ^b	5	7
B151-B207*	hCG nicked	10% hCG nicked free beta subunit; 12% hCG non-nicked; 2% hCG free beta subunit; 2% hLH; 5% hLH free beta subunit; <1% ^b	5	15
B152-B207*	choriocarcinoma hCG (C5) and choriocarcinoma hCG free beta subunit	100% hCG nicked (C5); 190% hCG free beta nicked (from C5); 10% hCG nicked (pregnancy); 5% hCG free beta nicked (pregnancy); 7% hCG (pregnancy); 6% hCG free beta subunit; <1% ^b	6	13
B406-A201*	hLH	<1% ^a	4	10
B505-B503*	hLH beta core fragment	<1% ^a	9	9
B408-B409*	hLH free beta subunit	29% hLH; <1% ^a	7	11

^a-(if not indicated) hLH, free beta hLH, hLH beta core fragment, hCG, free beta hCG, hCG beta core fragment;

^b-(if not indicated) the same as ^(a) plus nicked hCG and nicked free beta hCG (pregnancy).

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A quadratic discriminant analysis based on a cross-validation method in order to minimize bias correctly classified 91% of the normal pregnancy subjects and 80% of the EPL subjects.

The mixed effects analysis, testing for interactions between mole fraction of analyte and time since LMP found no significant time or group (EPL vs. normal) effects in the intact hCG assay. In the free beta subunit of hCG assay, there is a significant group effect but no time trend. In both the hCG beta core fragment measurement and the B152 measurement, both the hormone levels and the time trend from LMP were significantly different between the EPL and pregnancy groups. This study produced several important findings. It defined the spectrum of analytes which in both early pregnancy and EPL, thereby resolving the issue of which hCG analytes to measure in epidemiological studies in which EPL is the end point determination. More importantly, it illustrated for the first time that there are significant differences both in the pattern of analytes and the time course of their appearance between early normal pregnancy and EPL. This observation facilitates very early prediction of a distressed pregnancy by urinary hCG measurements at a time which would permit therapeutic intervention.

30 Immunoreactivity of different forms of hCG in the two
 IRMA's (B152-B207 and B109-B108)

The relative binding of three different forms of hCG (urinary hCG, pituitary hCG and choriocarcinoma hCG C5) has been characterized in the two hCG assays (Figure 3). Urinary non-nicked hCG and pituitary hCG are recognized nearly equally well by the two IRMAss, while C5

recognition is quite different. The B152-B207* assay is more sensitive to C5, which is to be expected because B152 antibody was developed and selected on the basis of higher affinity to C5. Urinary non-nicked hCG is
5 purified from the CR127 preparation of pooled normal pregnancy hCG. Conversely C5 is recognized with lower affinity by the B109-B108* assay, which has primary specificity for the hCG isoforms of later pregnancy.

10 We have developed a method to directly profile changes of hCG isoforms in serum or urine throughout pregnancy. Two IRMAs for hCG are employed, each based on monoclonal antibodies to different hCG epitopes. The B109-B108* assay is a commonly used intact hCG assay to the
15 heterodimeric-dependent epitope. A new assay, B152-B207*, is most likely sensitive to the carbohydrate portion of hCG carboxyterminal peptide. The same standard non-nicked hCG was used in both assays. Non-nicked hCG was employed since the B109 assay reacts
20 poorly with nicked forms of hCG while the B152 assay does not discriminate between nicked and non-nicked forms of the hormone. The B152 assay detected with greatly enhanced sensitivity hCG isoforms which appear earlier in pregnancy than isoforms measured by the B109
25 assay (O'Connor et al. 1998). Prior to development of the new immunometric assay system described in this report, it was not possible to readily discern the changes in hCG isoforms from very early pregnancy to mid pregnancy. The only available procedure for examining
30 these changes was isoelectric focusing of every patient specimen followed by immunoassay of every focused fraction (Berger et al. 1993; Ulloa-Aguirre et al. 1990). The IEF pattern reflects the heterogeneity of the charged sugar, sialic acid which varies with the multi-
35 antennary structures of the carbohydrate moieties in which sialic acid is the terminal sugar. Although we do not yet know the precise nature of the isoform epitopes

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being measured, the evidence for carbohydrate discrimination is based upon the hyperglycosylated structure of the immunogen, C5, used to develop the B152 monoclonal antibody and the antibody's reactivity with the hCG isoforms found in the JAR choriocarcinoma cell line. C5 hCG was isolated from a choriocarcinoma patient and has been thoroughly characterized as to its protein and carbohydrate content and structure (Elliott et al. 1997). It has been shown that C5 (and hCG from other choriocarcinoma subjects) differ in the protein moiety mainly by the presence of an increased number of nicked sites and by increased glycosylation relative to the hCG of normal pregnancy. In comparison with the hCG of normal pregnancy, choriocarcinoma derived hCG has increased fucosylation of the N-linked biantennary oligosaccharides in the beta subunit. In addition, the O-linked oligosaccharides in preparation C5 (a form of hCG produced from a single patient with choriocarcinoma) has a 100% tetrasaccharide core on the COOH-terminal region of the beta subunit. Normal mid pregnancy hCG has only 10-20% of this structure (Elliott et al. 1997). These observations, plus our own determination that the hCG synthesized by the JAR choriocarcinoma cell line provides a B152/B109 isoform ratio similar to that observed in early pregnancy, leads us to the conclusion that in very early pregnancy, the developing trophoblast secretes an isoform of hCG which resembles that produced in choriocarcinoma.

We have also tested recognition of pituitary hCG since its N-Asn carbohydrates differ somewhat from those of placental hCG, bearing a closer resemblance to those of hLH which have both sialic acid and sulfate groups (Birken et al. 1996). The carbohydrate structure of the b COOH-terminal portion of pituitary hCG is not yet known. Since B152 did not recognize any substantial differences between pituitary and placental hCG (Figure

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3), differences in N-Asn recognition are unlikely. In terms of the COOH-terminal carbohydrates, it appears that pituitary and placental hCG (mid-pregnancy isoforms) may be similar, assuming the O-linked carbohydrate on the C5 antigen is part of the epitope of B152.

Example 2: B152/B109 Ratio Predicts Pregnancy
Outcome

The B152/B109 ratio measured in urine samples throughout the pregnancy

The relative concentrations of hCG isoforms in 103 normal pregnancy urine samples (5-39 weeks post last menstrual period - LMP) were determined by two immunometric assays (B152-B207* and B109-B108*). Both because of the wide range of hCG concentrations in different samples, even at the same gestational age, and because neither of the assays is totally specific for the two (or more) families of hCG isoforms present, we find that presenting the data as a ratio of the observed two isoform groups more clearly delineates the change in isoform content as pregnancy progresses. This calculated ratio is shown in Figure 4. In weeks 5-8 of pregnancy, the ratio of B152/B109 isoforms ranged between 6.2 and 1.3, indicating a predominance of the B152 isoform(s) in early pregnancy. During the 10 to 12 week period, the ratio ranged from 1 - 0.2, indicating that an inversion in hCG isoform content is occurring as pregnancy progresses. This decline in the ratio continues, ranging from 0.54 - 0.08 in the 15 - 18 week period and reaching an inflection point at 29 weeks. At that time, the ratio reached a value of around 0.06 after which the ratio displayed a rise to a range of 0.2 - 0.07 in the 37 - 39.5 weeks of gestation time period.

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Statistical analysis involved fitting the log transformed ratio data to second and third order polynomial regression models. Since the third order term was not significant (likelihood ratio $\chi^2(1)=1.32$, $P=0.25$), the second order model was used ($r^2=0.793$). The log B152/B109 ratio reached an inflection point at LMP=29 weeks, based on this model.

The B152-B207* values reflect a measurement of the B152 isoform in terms of later pregnancy hCG equivalents, not in absolute quantities. It must be emphasized that the "absolute" concentrations measure in the B152 assay cannot be compared with the results of the B109 assay on an equimolar basis since the potency of the hyperglycosylated isoform is much higher in the B152 assay vis-à-vis the standard, i.e. normal later first trimester pregnancy hCG. The actual molar values of this isoform are on the order of tenfold less than those recorded in the assay. For this reason we have chosen not to analyze absolute molar quantities of the two analytes but only the ratio of the two measurements.

Even in normal pregnancy, the hCG values obtained vary widely according to the characteristics of the immunological reagents employed (Cole and Kardana, 1992; Cole et al. 1993). We hypothesize that the two assays described in this report primarily detect hCG isoforms at opposing ends of this spectrum, each primarily recognizing a subset of closely related molecules in the continuum of early to later pregnancy hCG molecular forms.

We have retained the use of normal pregnancy hCG as the standard in B152-B207* assay, despite its decreased affinity in this antibody configuration. The reasons for this include the limited and unrenewable supply of C5 (which was isolated from the urine of a single

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The B152/B109 ratio in matched serum/urine samples in the first and third trimesters of pregnancy compared with hCG from JAR cells.

5 The B152/B109 ratio in serum is analogous to that found
in matched urine samples and undergoes a similar change
as pregnancy progresses (Figure 5). The B152/B109 ratio
in the cell supernatant from JAR cells (a
choriocarcinoma derived cell line) was similar to that
10 of early pregnancy.

The B152/B109 ratios of both serum and urine hCG
concentrations are significantly higher in the first
trimester as compared to the third trimester of normal
15 pregnancies (Table 2). Significant differences between
serum and urine hCG concentration ratios as well as log
transformed ratios in early (5-6 weeks) and late (36-39
weeks) gestation were evaluated by paired t-tests (Table
3). In both the first and third trimesters, urinary
20 B152/B109 ratios were significantly higher than serum
ratios, indicating that there was a preferential
clearance of the B152-recognized isoform into urine,
regardless of the relative concentrations of the two
isoforms.

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TABLE 2.

Analysis of the B152/B109 ratio in serum and in urine in
the first vs third trimesters of pregnancy.

Measure	T-test (df)	P
Serum, ratio B152/B109	t (11) = 6.65	0.0001
Serum, log(ratioB152/B109)	t (23) = 21.61	0.0000
Urine, ratio B152/B109	t (11) = 4.64	0.0007
Urine, log(ratioB152/B109)	t (15.7) = 16.85	0.0001

TABLE 3.

Analysis of the B152/B109 ratio in serum vs urine in the first and third trimesters of pregnancy.

Gestational age	Measure	Paired-t (df)	P
5-6 weeks	Ratio B152/B109 Log(ratioB152/ B109)	t(11)=3.25 t(11)=6.25	0.0077 0.0001
36-39 weeks	Ratio B152/B109 Log(ratioB152/ B109)	t(10)=5.47 t(10)=7.14	0.0003 0.0001

The B152/B109 ratio in urine samples from IVF patients

In urine samples from IVF patients (1-4 weeks post embryo transfer - ET) the B152/B109 ratio was again between 2-8 and decreased as pregnancy progressed (Figure 6), similar to that observed in natural conceptions. The effect of pregnancy duration with respect to outcome variables could best be represented by a linear or quadratic function. ANCOVA models including the second order week were fitted to the general equation: Outcome= (effect of time post ET)+(effect of diagnosis). After an appropriate ANCOVA model was determined, the least square means (adjusted for week post ET effect) were compared among the normal pregnancy, ectopic pregnancy and spontaneous abortion populations (Table 4). The log transformed values of both B109-B108* and B152-B207* measured hCG forms discriminated both ectopic pregnancy and spontaneous abortions from normal pregnancy (P=0.0001). The ratio of the log transformed values discriminated abortion from normal pregnancy (P=0.016). However, neither the ratio of B152/B109 nor the log of that ratio discriminated either of the pregnancy disorders from normal pregnancy.

A significant number of spontaneous abortions and ectopic pregnancies occur in IVF pregnancies. We did not find a difference in the ratio of the isoforms between either of these two categories as compared to normal controls, possibly a consequence of low statistical power. However a significant difference was found between the B152 hCG isoforms levels in normal pregnancy and spontaneous abortion. This supports our previous finding in early pregnancy loss, where diminished or absent levels of the B152 isoforms characterized an early pregnancy loss (O'Connor et al. 1998).

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the B152-B207* assay, with a corresponding value of 148 fmol/ml in the B109-B108* determination. Six of seventeen samples in serum had detectable levels, with 4/6 having a higher value in the B152-B207* assay. Of the 15/28 positive urine samples however, 14/15 had higher levels in the B152-B207* assay than in the B109-B108* assay, with the highest hCG value being 20000 fmol/ml in the B152-B207* assay and 18715 fmol/ml in the corresponding B109-B108* assay. Due to the small sample size, no statistical treatment was performed on this data, but even in these post-treatment patients the B152/B109 ratio was ≥ 1 , which corresponds to the early pregnancy hCG isoform ratio.

The specimen limitations discussed above precludes our reaching any definitive conclusion on the analysis of trophoblastic disease samples. However it appears as might be anticipated that the B152 assay is more sensitive than B109 assay in detecting hCG immunoreactivity in the blood and in the urine of trophoblastic disease patients, even after treatment.

Chromatography of First Week of Gestation Pregnancy Pool. In order to determine whether the B152-B207* assay recognized other forms of hCG associated immunoreactivity in addition to the intact hCG molecule, specimens were pooled. FPLC on tandem Superose 12 columns followed by immunoassay of the fractions for all of the characterized forms of hCG revealed that only the intact hCG molecule (or hCG free beta subunit) gave a signal in this assay (See Figure 7). There were no lower molecular weight fragments identified by the B152-B207* assay. The hCG free beta analyte was measured in urine described in Figure 2 and was found to make a negligible contribution to over all hCG immunoreactivity in these specimens.

Serum and urine specimens were analyzed using two assays, B109-B108* and B152-B207*, which recognize the difference in molecular isoforms of hCG. See Table I. The *in vitro* bioassay for hLH/hCG is described above. (See Figure 1). The immunometric assay employs 96-well

Serum and urine specimens were analyzed using two assays, B109-B108* and B152-B207*, which recognize the difference in molecular isoforms of hCG. See Table I. The *in vitro* bioassay for hLH/hCG is described above. (See Figure 1). The immunometric assay employs 96-well

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profile of healthy normal pregnancies and unsuccessful pregnancies from failed IVF implantations: 1) the time at which the B152/B109 ratio falls below 1.00 is no different in healthy normal and unsuccessful IVF pregnancies; 2) the time at which the B152/B109 ratio declines by 1/3rd from first trimester maximum levels is no different in healthy normal and unsuccessful IVF pregnancies. Of course, the objective is to provide an empirical basis from which clinicians may counsel their patients. Thus, it is important to adopt a logistic model for this component of the data analysis. With pregnancy success as the outcome, logistic models allow the estimation of the (symmetrical) hypothesis of increase in risk of pregnancy failure for each additional week where either the B152/B109 ratio has failed to decline by one third from first trimester baseline maximum values or the B152/B109 ratio has failed to become less than 1.00 (measured in weeks). The logistic model enables specification of the time at which results indicate a particular pregnancy exceeds an a priori defined likelihood of failure, given assay data regularly available during pregnancy, and allows incorporation of other risks for pregnancy failure in the same data analytic framework to assess the relative contribution of threats to pregnancy loss. The Cox proportional hazard model may be used to examine predictors of the crossover rates. Mixed effects models can also analyze repeated measures of the B152/B109 ratios taken during entire cycles. These models are particularly useful since they allow inclusion of incomplete and imbalance data (i.e. data with missing values and unequal timing of data collection), to estimate effects of time-varying covariates, to model dependency structure of repeated measures and to model possible heterogeneity of the ratio measures within each experimental group.

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structures. Using the already developed scheme of concentration and immunoaffinity extraction of urine, hCG molecules are isolated from urine collected from women in early pregnancy for both protein and carbohydrate analyses. According to one approach,

carbohydrate analyses. According to one approach, molecules are isolated from HPLC fractions, digested with proteases before and after reduction of disulfide bonds, examination of the resultant peptides by mass spectrometry and/or sequence analysis, isolation of

combination of specific glycosidases and retention times on specialized anion exchange columns as compared to know branch-chain oligosaccharide standards. In a similar approach, the final purification stage for the isolated hCG isoforms is SDS gel electrophoresis. Both

similar approach, the final purification stage for the isolated hCG isoforms is SDS gel electrophoresis. Both protease digests and glycosidase digests are performed on the blotted and cutout band. This method results in greater purity of the protein and less artifactual

protease digests and glycosidase digests are performed on the blotted and cutout band. This method results in greater purity of the protein and less artifactual errors due to contamination by carbohydrates which are not in the purified protein but are derived from outside contaminants.

errors due to contamination by carbohydrates which are not in the purified protein but are derived from outside contaminants.

Carbohydrate compositional analyses and oligosaccharide

Carbohydrate compositional analyses and oligosaccharide branched chain identifications. The MALDI TOF mass spectrometric method may be used to confirm oligosaccharide structures by using specific

spectrometric method may be used to confirm oligosaccharide structures by using specific glycosidases on the glycopeptides and determining the change in molecular weight as the sugars are digested off the glycopeptide. Only the hCG beta COOH peptide

change in molecular weight as the sugars are digested off the glycopeptide. Only the hCG beta COOH peptide can be expected to contain O-linked sugar moieties. These are of special interest since it is thought that B152 has significant reaction with this region. The

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5 Example 3: B152 and B151 immunoreactivity in non-trophoblastic malignancy.

35 Experimental Discussion

In the course of these studies, a potentially important new signal was observed in the urine of women early in pregnancy, namely an epitope of a form of hCG which may

indicate the likely success of carrying a pregnancy. Likewise, absence of this signal may indicate that EPL will occur. Since EPL can be a very sensitive marker of environmental toxins (Hakim, R. B., et al., 1995) and is frequently used as an epidemiological marker of exposure, the finding of this epitope provides a powerful tool for monitoring the safety of the environment. In addition, this assay facilitates increasing the success rate of IVF infertility programs since the predictive value of the new measuring system would rapidly indicate successful approaches. Described herein is the novel and completely unexpected finding that successful pregnancies display a high content of unique isoforms of hCG that are maintained for the first few weeks of pregnancy and then rapidly decline as pregnancy progresses. Based on properties of the immunoassay system, it is hypothesized that these hCG isoforms may be hyperglycosylated. This is a striking observation never reported nor suspected earlier. Carbohydrate analyses (Elliot, M., 1997) demonstrate that C5 hCG employed as immunogen for antibody B152, contains two times the monoantennary content and three times the tri-antennary content of branch chain sugars as compared to the CR series of natural pregnancy urinary hCG. In addition, the O-linked carbohydrates are mostly tetrasaccharide instead of disaccharide in C5 as compared to CR 127 hCG. (CR 127 hCG is similar to the WHO preparation, the third international hCG standard, which was CR 119 hCG, prepared by Canfield and Birken twenty years ago but still in use today) (Birken, S., et al., 1991a). B152 recognizes C5 hCG much better than nicked CR127 hCG or non-nicked CR 127 hCG (Birken, S., et al., 1993). In addition, JAR cell type hCG is known to contain a similar array of carbohydrate moieties. It was found to be recognized by B152 similar to the early pregnancy isoforms in healthy pregnancies. The observation that the hCG isoform produced by JAR cells

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in culture (B152/B109 ratio) is similar to that found in early pregnancy hCG isoforms supports the hypothesis that the production of a type of hCG with a particular glycosylation pattern is a prerequisite for a viable pregnancy. This glycosylation pattern is not characteristic of the hCG of later pregnancy.

A variety of pregnancy disorders are testable. One category of patients consists of those women who experience a high rate of recurrent abortions. Even in populations with no known fertility problems, the total rate of pregnancy loss is 32% (EPL plus clinically recognized abortion) (Wilcox, A. J., et al., 1988). The risk of recurrent abortion increases with the number of spontaneous abortions experienced in the past, reaching an incidence of 32% after three consecutive abortions. (Hill, J. A., and Anderson, D. J., 1990). Probable causes of recurrent spontaneous abortion, comprising genetic, infectious, hormonal imbalance, or immunologic factors can be established in less than 60% of all spontaneous abortions, leaving 40+% of spontaneous abortions with a completely unestablished etiology. These facts, taken together with reports establishing that the administration of exogenous hCG can be an effective therapy in subjects with a history of recurrent spontaneous abortion (Quenby, S., and Farquharson, R. G., 1994; Harrison, R. F., 1985) lends support to the hypothesis that a disproportionate production of the ineffective isoforms of hCG in early pregnancy is a causal factor in both early pre-clinical loss as well as in spontaneous abortion.

A second category includes women undergoing embryo transfer. These patients provide several distinct advantages: The patients undergoing this procedure are not treated with crude hCG preparations, making measurement of hCG isoforms easy and decisive since all

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hCG forms derive from the embryo none from any injected hCG preparations. Second, is the opportunity to monitor the nature of the isoforms from day 9 of a successful pregnancy. Third, is the ability to obtain large volumes of urine to purify the early pregnancy isoforms to determine their structures. Fourth, since pregnancy loss is from 50% to 70% in this population, the loss can be defined as due to lack of the essential hCG isoform recognized by B152 or due to other causes. Comparison of early pregnancies in populations of women not undergoing *in vitro* fertilization procedures with those undergoing embryo implantation can, thus, assess whether pregnancy loss situations present similar or different patterns of hCG isoforms during the process. The mechanism of pregnancy loss in the general population as compared with the much higher rate of embryo loss in IVF programs may be different. Additionally, it has been established that the hCG produced in choriocarcinoma has differences in carbohydrate structures, sialic acid content and biological activity (Wide, L., and Hobson, B., 1987; Elliot, M., et al., 1997; Hussa, R. A., 1987). Since B152-B207* assay incorporate monoclonal antibodies raised against an immunogen derived from choriocarcinoma, specimens may be evaluated from patients with gestational trophoblastic disease in order to determine whether the above assays recognize the hCG produced in these conditions with greater sensitivity and specificity than do assays based on the hCG of normal pregnancy, as is apparently the case for the hCG produced in testicular and ovarian cancer.

There are few reports of changes of carbohydrate content of hCG-related molecules during pregnancy. Blithe and colleagues studied free alpha subunit of hCG whose carbohydrate content differs from that of alpha within hCG by additional carbohydrate antennae and fucose. The carbohydrate of free alpha becomes increasingly complex

in terms of more branches and higher content as pregnancy proceeds. It has also been reported that the quantity of fucose increased in both hCG and in free alpha as pregnancy proceeded (Skarulis, M. C., et al., 1992). Thus, the literature indicates increasing content and complexity of carbohydrate of hCG and free alpha subunits. However, immunological data using the B152 monoclonal antibody, implies a progression to simpler carbohydrate content during pregnancy. Since the beta COOH-region's O-linked carbohydrates may be involved in the epitope recognized by B152, it is conceivable that the carbohydrate structures of this region may be altered in a different pattern from the N-linked glycans studied by Blithe and colleagues (Skarulis, M. C., et al., 1992; Blithe, D. L., and Iles, R. K., 1995). Data from Skarulis et al. indicate that heterodimeric hCG may contain additional fucose but do not provide data that this late pregnancy hCG becomes hyperglycosylated as does free alpha.

Other studies indicated that the forms of hCG during EPL likely differ in biological activity from those hCG isoforms in successful pregnancies (Ho, H.-H., et al., 1997). The *in vitro* bioassays employed in those studies are unsuitable for large-scale studies and are not as reliable as the immunoassays described herein. Furthermore, it is likely that *in vivo* assays may give different results since *in vitro* and *in vivo* assays sometimes give completely disparate results. In this case, *in vivo* and clearance assays are most important in order to identify whether the hCG isoforms are truly more potent in the whole animal and to identify the reasons for the increased potency. Thus *in vitro* and *in vivo* bioactivities of the early pregnancy isoforms of hCG are highly significant.

Carbohydrate differences is a widely accepted

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explanation for variations in biological to immunological ratio such as the forms observed by various studies of EPL (Ho, H.-H., et al., 1997). Various studies (Grotjan, H. R. J., and Cole, L. A., 5 1989; Hoermann, R., 1997; Stanton, P. G., et al., 1993; Szkudlinski, M. W., et al., 1995, Thotakura, N. R., et al., 1994; Szkudlinski, M. W., et al., 1993), have shown that sialic acid differences are an explanation for such heterogeneity in biological activities of glycoprotein 10 hormones. These studies have also confirmed the dogma that *in vitro* biological activities can yield the opposite results from *in vivo* studies because of altered metabolic clearance rates in the latter studies. Thus, more acidic (more highly sialylated) forms of 15 gonadotropins are more biopotent in the whole animal because of prolonged circulating half-lives. The same molecules may appear less potent in *in vitro* assays due to greater acidity, greater negative sialic acid content. Hoermann et al. (Hoermann, R., et al., 1997) 20 demonstrated the exclusion of many of the acidic circulating hormone forms from the urine, thus, prolonging their half-lives. The pI pattern of normal pregnancy as well as trophoblastic cancer hCG in serum is quite different from that of urine. Since the 25 studies described herein indicate that EPL hCG isoforms have reduced *in vitro* biological activity, this finding cannot be explained solely by what is known of biological activity and sialic acid content. Early pregnancy isoforms recognized by monoclonal antibody 30 B152 may be more potent *in vivo* by virtue of prolonged half-life they may then display increased signal transduction at the receptor as well. This may be explained by a hyperglycosylated form of hCG which is not hypersialylated. In this case, the extra sugar 35 portion would help prolong circulating half-life of a more basic pI form of hCG which also has increased *in vitro* bioactivity.

Example 4: Diagnosis of Gestational Trophoblast Disease.

5 An important application of the B152 (early hCG
isoform)/B109 (late hCG isoform) ratio analysis
described herein above is in the very early (and facile)
diagnosis of gestational trophoblast disease. Examples
of gestational trophoblast disease include
choriocarcinoma or hydatidiform mole. In normal
10 pregnancy, the ratio of B152/B109 of the two isoforms of
hCG rapidly decreases, eventually inverting. In
gestational trophoblast disease including
choriocarcinoma or hydatidiform mole, the ratio is
initially higher than found in normal pregnancy, but
15 does not diminish during the course of the apparent
pregnancy. This approach provides a highly sensitive
and specific diagnostic marker for gestational
trophoblast disease.

20 Other pregnancy disorders in which hCG levels are
abnormally high or abnormally low include Down's
syndrome or other aneuploid pregnancies, ectopic
pregnancy, preeclampsia, and intra-uterine growth
retardation. Because the hCG production in these
25 conditions is quantitatively abnormal compared with
normal pregnancy, an altered ratio of the hCG isoforms
identified by B152 (early hCG isoform) and B109 (late
hCG isoform) can be detected.

30 Thus, the dual isoform analysis (B152/B109) further
provides a method for diagnosing pregnancy disorders and
gestational trophoblast disease.

Materials and Methods

35

Hormones

The non-nicked hCG isolated from the CR127 preparation

of hCG was used as a standard in both assays (Birken et al. 1993). The pituitary hCG was isolated as described (Birken et al. 1996). C5, a 100% nicked hCG having extra sugars on both N- and O-linked carbohydrate moieties, purified from the urine of a choriocarcinoma patient (Elliott et al. 1997), was supplied by Dr. Laurence Cole (Yale University School of Medicine). Although the C5 immunogen used in the development of B152 antibody was 100% nicked hCG isoform (i.e. had cleavages in the peptide backbone of loop 2 of the b subunit) the antibody did not discriminate nicked from non-nicked hCG (O'Connor et al. 1998).

The same serial dilutions of non-nicked hCG, pituitary hCG and C5 were used for binding characterization in hCG assays. Hormone concentrations of initial stock standards solutions were determined by amino acid analysis.

Immunoradiometric assays (IRMA)

The methodology used in the construction and validation of the ¹²⁵I-B109-B108* assay has been fully described elsewhere (O'Connor et al. 1988). The B152-B207* assay has also been characterized (O'Connor et al. 1998). Both assays were performed with a slight modification of the published procedure: the capture antibody was adsorbed onto the wells of microtiter plates (Immulon IV, Dynatech, Chantilly, VA) by incubating a 5 µg/ml solution (B109-B108* assay) or 25 mg/ml solution (B152-B207* assay) in coating buffer (0.2 M bicarbonate, pH 9.5) overnight at 4 C. The coating antibody solution was aspirated, the plates washed (wash solution: 0.9% NaCl, 0.05% Tween 20) and blocked with a 1% solution of BSA in

35 The first antibody is a capture, the second antibody with an asterisk is an iodinated detection antibody.

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PBS with 0.1% sodium azide. Following incubation with the BSA solution (minimum 3 hours at room temperature) the blocking solution was removed, the wells again washed with wash solution and 200 ml/well of the appropriate hCG standards were added in phosphate buffer B (PBS with 0.1% bovine gamma globulin and 0.1% sodium azide). After overnight incubation at 4 C, the plates were again aspirated and washed. The 200ml (50,000 cpm-100,000 cpm) of ^{125}I - labeled antibody was added to the wells which were again incubated for 24 h at 4 C. The tracer was aspirated, the plates washed with wash solution, the individual wells placed in glass tubes and the radioactivity determined in a Packard Cobra gamma counter. Doses were determined by interpolation from a smoothed spline transformation of the data points.

All samples were stored frozen at -20 C prior to assay. Because extreme values of sample pH may interfere with antibody binding, the urine pH was adjusted with 1.0M Tris (pH 9.0), 50 μl /ml urine prior to assay, so that the final pH was in the range of 7-7.4 (O'Connor et al. 1988). Intra-assay variation was 6% for both assays, inter-assay variation was 12% for B109-B108* and 13% for B152-B207* assays. Sensitivity (least detectable dose) defined as +2SD from the zero calibrator, was 1 fmol/ml for the B109-B108* assay and 2.2 fmol/ml for B152-B207* assay.

Patients samples

Urine samples from IVF patients were a gift from Dr. L. Cole. They included spontaneous abortion (n=14, range of gestational age 1.8-4.1 weeks from ET- embryo transfer), ectopic pregnancies (n=7, gestational age 2.3-4 weeks) and normal pregnancy controls (n=65, encompassing the range 0.6 to 5.4 week from ET). Some of the normal pregnancy urine samples throughout the pregnancies were

also obtained from Dr. Cole. Others were obtained from the clinical practice of collaborating physicians at Columbia Presbyterian Medical Center (CPMC) (Total n=103). Matched serum/urine samples from the first (n=12) and the third (n=11) trimesters were provided by Dr. Amalia Kelly at CPMC. Trophoblast disease serum (n=17) and urine (n=28) samples were obtained from Dr. Cole, but were collected by Dr. Edward Newlands (Charing Cross Hospital, London, UK). All specimen collection protocols were approved by the appropriate Institutional Review Board.

Statistical Analysis

Polynomial regression models of log transformed hormone ratios were used to describe the relationship between the change in ratio as a function of gestational age in normal pregnancy. A paired t-test was used to evaluate the relationship between matched serum and urine hormone ratios. Analysis of covariance (ANCOVA) was used to describe the time adjusted relationship of hormone values in ectopic pregnancy and spontaneous abortion to those of normal pregnancy.

Urine Processing.

Twenty-four hour urine samples are collected from women undergoing embryo transfer as well as women in early natural pregnancy. The urine is refrigerated during the collection procedure. After delivery of the urine to the laboratory, sodium azide is added to 1g/liter. Women undergoing in vitro embryo transfer are not pre-treated with hCG. Thus, all hCG which appears in their blood or urine is derived from the embryo (except for the small amounts of pituitary hCG present in all people). Raw urine is freed from particles by centrifugation followed by Pellicon filtration through a 0.45 micron membrane. Next, the procedure is to

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concentrate the urine with a Pellicon (Millipore) system which concentrates as much as 30 liters to 500ml overnight (4°C) using a 3,000 MW cutoff membrane. Smaller volumes can be concentrated in less than two hours. Next, the urine is desalted and delipidated by passage through a large volume of Sephadex G25 in 0.1 M ammonium bicarbonate. This step greatly increases the binding of CG to immunoaffinity columns. The desalted urinary concentrate is next size fractionated on the Pharmacia HiLoad Superdex 200 and the hCG and hCG subunit peaks are identified by specific immunoassays (O'Connor, J. F., et al., 1994) and the appropriate fractions are pooled and dried. The hCG and hCG subunits are purified from the gel filtered urine concentrate by immunoaffinity on insolubilized hCG antibody columns as described but with the use of either 4M guanadine (0.1M tris acetate, pH 5) or ammonium thiocyanate as eluant to decrease loss of sialic acid from the hormone. Alternatively, hCG is purified by conventional chromatographic procedures, anion exchange and hydrophobic chromatography. The subunits are separated on reverse phase HPLC using a 0.01M sodium phosphate, pH 5 buffer and acetonitrile, after incubation in 4M guanadine, 0.1M tris acetate, pH 5. A third method is final purification and separation of the hCG subunits on SDS PAGE electrophoresis followed by electroblotting to PVDF. The PVDF band can be subjected to protease digestion to release peptides and glycopeptides which can be separated on reverse phase HPLC in neutral pH 5 buffers.

Separation of Glycopeptides from Isolated hCG subunits.

To facilitate isolation of the glycopeptides from the hCG subunits, the subunits are both tryptic digested and the products of digestion are separated on reverse phase HPLC (using a pH 5 buffer). This procedure results in removal of the large beta COOH-terminal peptide which

contains O-linked sugars. It also releases small, non glycopeptides from both subunits (Pollak, S., et al., 1990, Birken, S., et al., 1987; Birken, S., et al., 1986). Next, the main disulfide-linked core of each hCG subunit, is reduced and carboxymethylated, and separated on reverse phase HPLC at pH 5. At this stage, large peptides are isolated, including the glycopeptides. Each separated glycopeptide is redigested with trypsin and re-separated on HPLC at pH 5. These glycopeptides are next employed for two different methods of sugar chain analysis. One method is the approach of releasing the oligosaccharides by enzymatic digestions using PNGase F for the N-linked glycans. The released glycans can be separated from the peptides by ethanol precipitation, desialyated with neuraminadase, and separated directly on a Dionex Carbopac PA-100 column. Oligosaccharide standards are available from Dionex, Oxford Glycosystems and other companies for calibrating column elution times for various glycans (Hardy, M. R., and Townsend, R. R., 1994, Rohrer, J. S., et al., 1993, Weitzhandler, M., et al., 1993; Townsend, R. R., et al., 1989). Confirmation of the released structures is obtained by performing carbohydrate compositional analysis of eluted glycan peaks as well as performing digestions with specific glycosidases and rechromatographing the modified glycan on the Dionex system (Hardy, M. R., and Townsend, R. R., 1994; Rohrer, J. S., et al 1993; Weitzhandler, M., et al., 1993; Townsend, R. R., et al., 1989; Townsend, R. R., et al., 1991; Townsend, R. R., et al., 1989; Hardy, M. R., and Townsend, R. R., 1989; Townsend, R. R., et al., 1988; Hardy, M. R., et al, 1997; Hardy, M. R., and Townsend, R. R., 1988; Dionex, 1997; Spellman, M. W., 1990; Kumarasamy, R., 1990). The newly modified glycan can be observed to elute at the same time as the appropriate standard oligosaccharide and, in addition, the released monosaccharide can frequently be identified as well (Dionex, 1997). Structure determination is

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facilitated by the use of specific glycosidases for branch chain cleavage as well as for digestion of individual sugars from each of the branch chains. For example, Endo H cleaves high mannose type and hybrid oligosaccharide chains while glycosidase Endo F2 cleaves biantennary complex types and PNAase F cleaves tri and tetra-antennary chains down to the N-Asn bond.

Competitive receptor binding and in vitro bioassay.

Bioassays are performed with recombinant-engineered CHO cells transfected with the human receptor to LH/CG Cells are maintained in Ham's F-12 medium, 4mM Glutamine, 400ug/ml G418 (Gibco), 5% fetal calf serum, 100IU/ml penicillin, 100ug/ml streptomycin. The cells are removed from the flask surface by versene only.

A competitive receptor assay constructed as follows: The receptor binding assay mixture contains 100 ul of the appropriate dilution of serum/urine samples or hCG dilutions for standard curve, 100ul of ¹²⁵I-hCG (50,000-100,000cpm) in buffer A(PBS/0.1%BSA) and 100ul of CHO cells (2x10⁵ cells in PBS). The mixture is incubated at 37°C with slight shaking followed by centrifugation for 10 minutes at 750xg. The supernatant is aspirated and the cell pellet is counted in gamma-counter.

In vitro bioassay. Transfected CHO cells are seeded (200,000 cells/well) into a 24 well plate in culture medium and incubated for 2-3 days until the cells reach confluence. Non-transfected CHO cells are included to monitor non-specific response. The medium is removed and replaced with medium containing 1 mM isobutylmethylxanthine with appropriate dilutions of tested serum or urine. The plates are incubated at 37°C for two hours. The supernatant is removed, and the wells washed with Hank's balanced salt solution. The intracellular cAMP is extracted with 95% ethanol, which

is diluted 1:5, (or up to 1:40, depending on cAMP content) in assay buffer provided by the cAMP kit (New England Nuclear). cAMP assay is performed according to manufacturer's instructions. Response is normalized to well protein content (BCA protein assay kit, Pierce, Rockford, IL).

In vivo bioassay is determined by the uterine weight assay in immature female mice, following the procedure of Wide and Hobson (Wide, L., and Hobson, B., 1987). The mice are injected subcutaneously with one third of the total dose of gonadotropin on three consecutive days and killed 72 hours after the first injection. Uteri are dissected free from mesentery, fat and oviducts, blotted to remove intrauterine fluid and weighed to the nearest 0.1mg. Five to ten mice are used at each of these dose levels. The hCG standard preparation used is a nicked hCG. This material may be run concurrently with specimens isolated from first and third trimester pregnancy. Sham saline injection may be used as a control. The response signal is the log mouse uterine weight.

Clearance of hCG isoforms. The clearance of hCG is determined in the rat. Blood (200ul/sample) is obtained at 0, 120, 240, 360 and 480 minutes post injection, from an indwelling catheter in an catheterized external jugular vein, following the procedure described by Newman et al. (Newman, C. B., et al., 1985) and Brown and Hedge (Brown, M. R., and Hedge, G. A., 1972). Briefly, adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington MA), wt 175-225g, are given free access to food and water. Rats are handled for acclimatization for one week after arrival, and several days before the hCG infusion, the rats are cannulated under pentobarbital anesthesia. A 21 gauge stainless steel cannula is inserted into the one external jugular

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vein. The placement of the catheter allows for the collection of blood from the unrestrained, unstressed rat. After the animals have recuperated from the cannula implacment , an hCG isoform is injected (10 μ g/ml sterile saline) through the cannulated vein. Blood samples are obtained at the four time intervals listed above. The blood is allowed to clot and the serum separated and stored at -80°C for immunometric assays specific for different hCG isoforms.

Clearance rate of the isoforms of hCG from the circulation of the rat are estimated by computer fitting the concentration data to an equation of the general form:

Concentration = $Ae^{-\alpha t} + Be^{-\beta t}$ at time t; A and α are parameters of the rapid component and B and β are parameters of the slow component. The metabolic clearance rate (MCR) is calculated as $MCR = \text{Dose} / (A/\alpha + B/\beta)$ and the initial volume of distribution is calculated from $V_d = \text{Dose} / (A + B)$. The MCR is normalized to body weight for statistical analysis, which is performed using ANOVA with Duncan's range test for determination of significance (Cassals, J. W., et al., 1989).

Mice. The mouse species used in the experiments described herein are Balb/c mice, aged 12-20 weeks old and adult Sprague-Dawley rats of either sex. Mice used for the production of monoclonal antibodies through ascites and for the determination of *in vivo* biological activity as described. Balbc/c mice are used because hybridoma cell lines were developed using Balb/c splenocytes.

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